Temperature-responsive and degradable hyaluronic acid/Pluronic composite hydrogels for controlled release of human growth hormone

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Abstract

Temperature-sensitive hyaluronic acid (HA) hydrogels were synthesized by photopolymerization of vinyl group modified HA in combination with acrylate group end-capped poly(ethylene glycol)–poly(propylene glycol)–poly(ethylene glycol) tri-block copolymer (Pluronic F127). The synthesized HA/Pluronic composite hydrogels gradually collapsed with increasing temperature over the range of 5–40°C, suggesting that the Pluronic component formed self-associating micelles in the hydrogel structure. Upon prolonged incubation in a buffer medium, the micelles slowly degraded due to the hydrolytic scission of the ester linkage between the Pluronic and acrylate group. The mass erosion occurred much faster at 37°C than at 13°C, indicating that at the higher temperature, the ester linkage between the Pluronic and acrylate group might be more exposed to an aqueous environment and thus be more readily hydrolyzed due to Pluronic micellization. Incorporation of recombinant human growth hormone in the hydrogel resulted in a sustained release profile which followed a mass erosion pattern. © 2002 Elsevier Science B.V. All rights reserved.

Keywords: Hyaluronic acid; Pluronic; Degradation; Temperature-sensitive

1. Introduction

Synthetic and natural hydrogels have been widely used as novel biomaterials in diverse medical applications. Among them, stimuli-responsive polymers and hydrogels, which undergo a phase transition in response to external stresses like temperature [1–3], pH [4,5] and light [6,7], have received much attention over the past decade. In particular, temperature-sensitive hydrogels such as poly(N-isopropylacrylamide, NIPAAm) have been extensively studied for various applications such as drug delivery [8,9], cell culture [10,11], bioreactors [12] and diagnostics [13]. Most temperature-sensitive polymers showed a lower critical solution temperature (LCST) behavior in an aqueous solution [14,15]. A delicate balance of hydrophilicity/hydrophobicity in the polymer structure is responsible for exhibiting the LCST phenomenon [16]. A series of tri-block copolymers composed of poly(ethylene glycol)–poly(propylene glycol)–poly(ethylene glycol) (Pluronics) are another class of temperature-sensitive...
polymers, and they also demonstrate reversible sol–gel transition behaviors in aqueous solution [17]. Self-association of Pluronics with an increase in the temperature results in the gelation, which was attributed to the temperature-induced formation of micelles under the concentration range of above 20% (w/w) [18].

Hyaluronic acid (HA) is a naturally occurring polysaccharide composed of N-acetyl-D-glucosamine and D-glucuronic acid. HA is a major component of extracellular matrix in connective tissues and is particularly abundant in the vitreous and synovial fluids [19]. It plays a pivotal role in wound healing process in various tissues that requires the structural organization of the extracellular matrix for promoting cell differentiation and maintaining cell morphology [20]. Recently, HA has been clinically used as a visco-supplementation agent for ophthalmic and joint surgery [21,22]. Since HA is biocompatible and biodegradable, it has been popularly used as temporary scaffolds for tissue engineering or drug delivery devices for therapeutic agents [23]. For this purpose, HA was cross-linked to form a three-dimensional chain network (hydrogel) after chemically modifying HA with cross-linkable functional groups. A wide variety of polyfunctional cross-linking agents were used to form the hydrogel structure [24]. Alternatively, HA hydrogels were also prepared by polymerization of acrylate monomer conjugated HA by means of a chemical initiator or photo-irradiation [25]. It was demonstrated that in situ formed photo-crosslinkable hydrogels could be potentially used as delivery vehicles for various therapeutic proteins and cells.

In this study, it was attempted to synthesize thermally sensitive HA hydrogels based on vinyl group functionalized HA and Pluronic polymers. Carboxylic acid group of HA in the backbone was chemically modified with a vinyl monomer, N-(3-aminopropyl)methacrylamide, to produce methacrylated HA, and two terminal hydroxyl groups in Pluronic F127 was also end-capped with acrylate monomer. Methacrylated HA and di-acryloyl Pluronic were combined in different molar ratios in an aqueous solution and photo-polymerized under UV radiation. It was hypothesized that the resultant HA/Pluronic composite hydrogels would demonstrate thermally reversible swelling–de-swelling behaviors because the temperature-induced self-association of Pluronic component occurred in the hydrogel structure. The HA/Pluronic hydrogels were characterized in terms of short-term reversibility of temperature-dependent swelling–de-swelling behavior and long-term degradation/erosion behavior. Recombinant human growth hormone (rhGH) was loaded in the hydrogel to achieve a sustained release over a prolonged period.

2. Experimental

2.1. Materials

Sodium hyaluronate (HA) (MW: \(1.75 \times 10^6\)) was obtained from Pacific Co. (Seoul, Korea). Recombinant human growth hormone was donated from Dong-A Pharmaceutical Co. (Seoul, Korea). N-(3-aminopropyl)methacrylamide hydrochloride (APMMA) was purchased from Polysciences (Warrington, PA). 1-Ethyl-3-[3-(dimethylamino)propyl]-carbodiimide (EDC) and 1-hydroxybenzotriazole (HOBt) were purchased from Sigma Co. (St. Louis, MO). Triethylamine, (4-Benzoylbenzyl)trimethylammonium chloride and acryloyl chloride were purchased from Aldrich (Milwaukee, WI). Pluronic F127 ((EG)\(_{99}\)(PG)\(_{99}\)(EG)\(_{99}\)) was a gift from BASF (Ludwigshafen, Germany). All other chemicals were of analytical grade.

2.2. Synthesis of modified hyaluronic acid

HA (500 mg, \(2.85 \times 10^{-4} \) mmole) and APMMA (472 mg, 2.64 mmole, two-fold molar excess relative to \(-\text{COOH} \text{ in HA}\)) were dissolved in 150 ml of deionized water (pH 6.8). EDC (252.8 mg, 1.32 mmole) and HOBt (178 mg, 1.32 mmole) dissolved in 10 ml of a 1:1 mixture of dimethylsulfoxide (DMSO) and water were added into the above solution. The stoichiometric ratio of \(-\text{COOH} \text{ in HA}/\text{EDC/HOBt}\) was 1:1:1. The conjugation reaction was carried out for 1 day. The APMMA derivatized HA was dialyzed against deionized H\(_2\)O for 12 h (Spectra/Por\(_8\), Mw cut-off 10 000) and then was freeze-dried. Percent modification of HA by APMMA was determined by analyzing the \(^1\)H-NMR spectra. \(^1\)H-NMR spectra were taken with a Brucker.
DRX 400 spectrometer operating at 400 MHz. Chemical shift (δ) was measured in ppm by using 3-(trimethylsilyl)propionic-2,2,3,3-d₄ acid (TSP) sodium salt as an internal reference.

2.3. Synthesis of di-acryloyl Pluronic

Dried Pluronic F127 (20 g, 1.59 mmole) and triethylamine (0.55 ml, 3.98 mmole) dissolved in 200 ml of dichloromethane were charged in a 500-ml round-bottomed flask. Acryloyl chloride (0.25 ml, 6.36 mmole) was added in a drop-wise manner. The reaction mixture was stirred at 4 °C for 12 h and then at room temperature for 12 h. After filtration, diacryloyl Pluronic F127 macromer was purified by precipitation in an excess amount of diethyl ether and dried under vacuum for 1 day. The extent of acrylation was determined by ¹H-NMR.

2.4. Synthesis of hyaluronic acid/Pluronic hydrogels

Two different amounts of di-acryloyl Pluronic were dissolved in 12 ml of degassed deionized water containing 1% (w/w) methacrylated HA to obtain 10 and 15% (w/w) di-acryloyl Pluronic solution. (4-benzoylbenzyl)-trimethylammonium chloride was added as a photo-initiator to the mixture solution to obtain a final concentration of 3% (w/w) of the polymer. Each mixture was poured into a glass dish and cross-linked to form a hydrogel upon exposure to long wavelength UV (100 Watt, intensity 21.7 mW/cm²) by a UV source (UVP model B 100 AP, Upland, CA) for 25 min under nitrogen atmosphere. Disk shaped gels with a diameter of 1.2 cm were excised with a cork borer and immersed in deionized water to remove unreacted polymer. The disks were removed and dried first in air for 1 day followed by in a vacuum oven for 2 days.

2.5. Swelling studies

In order to examine temperature-sensitive behaviors of HA/Pluronic hydrogels, swelling ratios at different temperatures were measured. Swelling ratio was gravimetrically determined in triplicate by dividing the wet gel weight by the dry gel weight at each temperature. For the swelling experiments, gel disks with known dry weights were incubated in phosphate buffered saline (PBS, 10 mM, pH 7.4) at a specific temperature. Equilibration was carried out for 12 h before measuring the wet weight. In the oscillatory swelling measurements, gel disks were incubated in PBS and the temperature was varied between 13 and 37 °C in a step-wise function. The measurements were done in duplicate.

2.6. Degradation studies

Two dry gel disks were weighed and swelled in PBS (pH 7.4) solution at 13 and 37 °C for a desired period. At specified time intervals, the disks were freeze-dried, and then weighed. Mass erosion percent was determined by taking into account the dry weight difference before and after the incubation.

2.7. Protein release studies

For protein release studies, recombinant human growth hormone (rhGH) was used. A total of 5 ml of HA/Pluronic (1:15) mixture containing rhGH (31 mg) was photo-polymerized as described above. After washing the gels with PBS, they were incubated in 1 ml of PBS buffer medium (pH 7.4) at 13 and 37 °C. The amount of rhGH released in the incubation medium was determined by using a Coomassie Plus Protein Assay kit (Pierce, Rockford, IL). It was found that a Micro-BCA (bicinchoninic acid) protein assay method for rhGH was highly interfered with various degradation products from the gel. In contrast, the Coomassie assay was not affected.

3. Results and discussion

Methacrylated HA was prepared by conjugating a primary amine group of APMMA to carboxylic acids in HA by using EDC and HOBr as coupling agents. Fig. 1 shows ¹H-NMR spectra of the methacrylated HA. The substitution degree of HA was calculated by comparing the relative peak intensity ratio between two protons of the vinyl group in APMMA (=CH₂, 5.5 and 5.8 ppm) and three protons in the methoxy group of HA (–C=OCH₃, 2.1 ppm). The degree of substitution was 33%. The degree of
Fig. 1. $^1$H-NMR of methacrylated hyaluronic acid and di-acyloyl Pluronic.
substitution for di-acryloyl Pluronic F127 was also 235 determined by $^1$H-NMR in a similar way (Fig. 1). It 236 was 87% based on the relative peak ratio of acrylic 237 protons at each end of Pluronic ($=\text{CH}_2$, 6–6.5 ppm) 238 and three protons of methyl group of propylene 239 oxide unit ($=\text{CH}_3$, 1–1.2 ppm) in Pluronic. HA/ 240 Pluronic hydrogels were prepared by photo-poly- 241 merization of methacrylated HA and di-acryloyl 242 Pluronic at two different HA/Pluronic weight ratios 243 of 10 and 15% (w/w) in the presence of a photo- 244 initiator. Mechanical strength of the HA/Pluronic 245 hydrogels gradually increased with the photo-irradiation 246 time. It was empirically found that 25 min of 247 photo-irradiation time optimally produced soft and 248 malleable hydrogels sufficiently enough to handle. 249 Di-acryloyl Pluronic may act as a bi-functional vinyl 250 cross-linker between HA chains. Therefore pro- 251 longed photo-polymerization resulted in more exten- 252 sively cross-linked chain networks accompanied with 253 increasing the mechanical strength.

Fig. 2 shows the swelling ratios of HA/Pluronic 254 hydrogels prepared with two different weight ratios 255 of HA to Pluronic. The hydrogel with 10% (w/w) 256 Pluronic has higher swelling ratio than that with 15% 257 (w/w) Pluronic, but both of them show similar 258 temperature-sensitivities. Swelling ratio was deter- 259 mined after incubating the hydrogels in PBS buffer 260 (pH 7.4) for 12 h at different temperatures. There 261 was no apparent change in the wet weight of 262 hydrogels after 12 h incubation. However, this does 263 not mean that these hydrogel samples reached an 264 equilibrium state after 12 h incubation, because they 265 slowly eroded with time, as will be discussed later 266 (Fig. 2 shows pseudo-equilibrium swelling results at 267 different temperatures). It can be seen that the 268 swelling ratio continuously decreases with increasing 269 temperature. Particularly, the swelling ratio tends to 270 show a more sharp decrease around 20°C. The 271 results indicate that the local micellization of the 272 Pluronic component, which was induced by raising 273 temperature, occurred and played a critical role in 274 the de-swelling of the HA/Pluronic hydrogels. The 275

![Swelling ratios of HA/Pluronic hydrogels as a function of temperature at pH 7.4.](image)

Fig. 2. Swelling behaviors of two HA/Pluronic hydrogels as a function of temperature at pH 7.4.
self-association of relatively hydrophobic poly-(propylene glycol) middle block in Pluronic was likely to reduce the mesh size in hydrogels at higher temperatures, thereby decreasing the overall swelling ratio. Since Pluronic tri-block copolymers were presumed to cross-link HA chains inter- and intramolecularly, the thermally induced Pluronic micellization restricted the swelling tendency of HA chains. This is schematically shown in Fig. 3. It was reported that Pluronic F127 exhibited thermal gelation behavior above a certain concentration (20% (w/w)) in water [18]. Although 10 and 15% (w/w) Pluronic was incorporated within 1% (w/w) HA hydrogels in the present study, thermal transitions over a rather broad range of temperature could be still observed. This can be attributed to the effect of HA on the micellization of Pluronic. The presence of highly negatively charged HA in the vicinity of Pluronic component might change the critical concentration as well as the gelation temperature of Pluronic F127. It was well established that various additives such as salts, polymers, and surfactants influenced the gelation temperature of various Pluronic polymers [28,29].

In order to investigate whether HA/Pluronic hydrogels reversibly swelled and de-swelled against temperature change and to examine how fast the hydrogels responded, cyclic swelling and de-swelling of the hydrogels were induced by cycling temperatures between 13 and 40 °C in a step-wise function. As shown in Fig. 4, the HA/Pluronic hydrogels responded to temperature cycles. However, the thermal response of the hydrogels was not perfectly reversible, and swelling ratios tend to increase with the number of cycles. This change in the swelling ratio can be attributed to the partial chemical degradation of HA/Pluronic hydrogels by hydrolysis of...
ester linkage present in di-acryloyl Pluronic component. The gradual increment of swelling ratio was much more noticeable for HA/Pluronic (1:10) hydrogels than HA/Pluronic (1:15) hydrogels, suggesting that the hydrolysis of ester linkage was faster for the less cross–crosslinked HA/Pluronic (1:10) hydrogel that was more hydrated. In addition, it can be seen that the swelling ratios of the hydrogels increase to a greater extent after exposure to 37 °C than to 13 °C. This indicates that the effect of temperature on the hydrolysis of ester linkage was much greater than that of water hydration for the degradation of HA/Pluronic hydrogels. To further investigate long-term degradation behaviors, HA/Pluronic (1:10) hydrogels were incubated at two temperatures, 13 and 37 °C. Fig. 5 shows that HA/Pluronic hydrogels degrade much faster at 37 °C than at 13 °C, even though they were less hydrated with increasing temperature. At 37 °C, the mass erosion of HA/Pluronic hydrogel began to accelerate after day 5 with showing an initial lag period. In contrast, at 13 °C, no significant mass erosion of less than 90% occurred over the incubation period of 45 days. This result suggests that the hydrolytic scission rate of ester linkage was mainly affected by temperature and temperature-induced Pluronic micellization, not by the degree of hydration. It is conceivable that a cleavable ester linkage of acrylate group, end-capped to both terminal ends of Pluronic, might be more locally exposed to an aqueous environment at a higher temperature because of the self-association of Pluronic polymer chains. The thermally induced formation of Pluronic micelle domains in the hydrogel was likely to contract a HA chain network. Therefore, increased junction tension between the Pluronic and HA chains allowed the ester linkage to be cleaved more readily.

In-situ polymerizable temperature-sensitive hydrogels can provide a non-denaturing environment for delivering bioactive proteins in a sustained manner. Photo-polymerized degradable PEG hydrogels based on di-acrylated oligo-lactide derivatized PEG were previously used for delivering proteins [30,31]. Since both HA and Pluronic are known to be biocompatible, they have been widely used as drug delivery carriers. rhGH, which necessitates multiple injections for a long-term period in treating juvenile dwarfism, was used in this study to explore the sustained release capability of the HA/Pluronic hydrogels. While injectable rhGH sustained release formulation using biodegradable poly(lactide-co-glycolide) microspheres has already been commercialized, alternative delivery systems based on novel hydrogels are attracting much attention recently. rhGH (MW 22 000) was loaded within HA/Pluronic hydrogels by photo-polymerization and its release pattern in PBS solution was investigated at 13 °C and at 37 °C. In vitro release profiles of rhGH from HA/Pluronic hydrogel at the two temperatures are shown in Fig. 6. Two separate HA/Pluronic hydrogel batches demonstrate very similar release profiles at both of the temperatures, validating the reproducibility of the results.

Fig. 5. Degradation of HA/Pluronic (1:10) hydrogels as a function of time.

Fig. 6. Release profiles of human growth hormone from HA/Pluronic hydrogels.
rhGH release experiment. For both of the batches, there is significant difference in the rhGH release behavior between 13 and 37 °C. Initial burst releases of rhGH from the swollen hydrogels were observed at both temperatures: 31% at 13 °C and 24–26% at 37 °C after 1 day incubation. The burst release was likely to occur by the rapid diffusion of surface located and loosely entrapped rhGH in the hydrogel matrices upon incubation. After the burst releases, the HA/Pluronic hydrogels incubated at 37 °C exhibit a short duration of slow release followed by an accelerated release starting from days 3 and 4. On the other hand, the hydrogels incubated at 13 °C do not show such a rapid release. Considering that the HA/Pluronic hydrogels eroded more rapidly at the higher temperature as shown in Fig. 5, the accelerated rhGH release profile observed at 37 °C was clearly due to the degradation/erosion of hydrogel matrices. Gradual disintegration of the polymer chain network led to enlarged mesh size, and thus enabled the entrapped rhGH molecules to diffuse out more readily. After 12 days of incubation, the cumulative rhGH release percent reached 55% at 13 °C and 84–90% at 37 °C. The incomplete release at 13 °C for 12 day incubation was apparently caused by the slower degradation of the HA/Pluronic hydrogels. In general, the erosion controlled release profile of proteins was hardly achieved in popularly used biodegradable poly(lactide-co-glycolide) [PLGA] matrices. This is because the protein release kinetics from these matrices involve complicated protein stability events such as aggregation and non-specific adsorption. They often lead to unpredictable and uncontrollable release profiles [32].

Using more hydrophilic polymers of HA and Pluronic polymers in combination, which are more benign to proteins in terms of protein stability, is a good alternative way to achieve a sustained release locally as well as systematically. In-situ polymerized hydrogels are expected to provide more resistant micro-environments against protein aggregation and non-specific adsorption than hydrophobic biodegradable PLGA polymers, because they contain much larger amount of water in the matrices. In addition, in-situ polymerized hydrogels do not need any organic solvents for protein loading in contrast to PLGA matrices that requires an organic solvent. Use of organic solvent is very detrimental in preserving a correct protein conformation after encapsulation. Lastly, it should be noted that whether or not the released rhGH species had a native intact structure with retaining full biological activity was not examined in this study. Since the photo-polymerization process generated harmful free radicals that might lead to chemically altered rhGH structures such as oxidized and crosslinked rhGH, more detailed analysis would be necessary to confirm the structural integrity of released rhGH. This will be reported in the near future.

4. Conclusion

In conclusion, it was demonstrated that a new class of thermo-responsive hydrogels based HA and Pluronic was synthesized by photo-polymerization. HA/Pluronic composite hydrogels exhibited temperature-dependent swelling and collapse behaviors in aqueous solution, which was presumably caused by the local micellization of Pluronic polymer with increasing temperature. These hydrogels chemically degraded and eventually eroded as a result of hydrolytic scission of ester linkage existing in the structural unit of di-acryloyl Pluronic component. The mass erosion occurred much faster at a higher temperature, possibly because the ester linkage was more sterically exposed to hydrolysis reaction. rhGH release from in situ photo-polymerized HA/Pluronic hydrogels showed quite different profiles between 13 and 37 °C. rhGH release profiles were well correlated with mass erosion patterns, revealing that the protein release rate could be controlled by modulating the temperature. Thermo-responsive and degradable HA/Pluronic hydrogels can be potentially applied for delivery of macromolecules such as peptides and proteins.

5. Uncited references

[26]; [27]

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References


